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Evaluation of the Potential Therapeutic Anti-inflammatory, Anti-proliferative and Apoptotic Effects of *Viburnum tinus* Leaves Different Extracts.

Fatma H Salman [*a], Ekrami A Hassan [c], Mona A Mohamed [b], Soad A Sherif [a], Mona N Moharib [a] and Eman M Abd El Azeem[c]

[a]*Biochemistry and molecular biology department,TBRI,Giza,Egypt.* [b]*Medicinal chemistry department,TBRI,Giza.Egypt.* [c] *Biochemistry department,Faculty of science,Ain Shams university,Cairo,Egypt.*

Abstract

Viburnum tinus *(V.tinus)* is an Adoxaceae family medicinal plant rich in vital phytochemicals such as polyphenols, triterpenoids, flavonoids, coumarins, and iridoids. This study shows that *V. tinus* leaf extracts have anti-inflammatory, anti-cancer, and apoptotic properties. The plant leaves were dried and soaked in 90% MeOH to prepare the crude extract, which was fractionated by n-Hexane, CH_2Cl_2, and MeOH. Phytochemicals were detected in these extracts, and their anti-inflammatory, anti-cancer, and apoptotic activities were evaluated *in-vitro*. The highest content of flavonoids, tannins, terpenoids, and coumarins in the n-Hexane extract stimulates its anti-inflammatory activity, as evidenced by protein denaturation inhibition (94.60±3.90 %), proteinase inhibition (88.5±0.50 %), membrane stabilization (73.2±1.58 %), and inflammatory cytokine (INF- ɤ) inhibition (100±0.00 %) without any toxicity on blood cells. It also has a cytotoxic impact on HepG2, MCF7, and CaCO2 cell lines, with IC_{50} (29.7 2.58, 75.3 4.70, 29.1 0.59 µg/ml) and SI (10.2, 10.4, 4) on HepG2, MCF7, and CaCO2, respectively. The apoptotic impact of n-Hexane in HepG2 resulted in quantitative expression of the BAX gene, whichincreased significantly and the BCL2 gene, which decreased significantly.

Keywords: Viburnum tinus, anti-inflammatory, anti-cancer, apoptosis, DNA fragmentation, INF-gamma, pro and anti-apoptotic.

Introduction

Medicinal plants contain a diverse range of phytochemicals, which have been used for decades as safer and less expensive alternatives for millions of people, particularly in developing countries [1]. Theprevalence of bioactive phytochemicals such as alkaloids, flavonoids, tannins, and saponins as a possible route for disease prevention and therapy is a common denominator in all medicinal plants [2].

Egypt is a country rich in plant variety and has been asignificant hub for the use of traditional herbal medicine for many years. The government needs to improve planning techniques for the efficient use of these therapeutic plants and herbs locally, as well as their marketing and exporting globally [3].

Herbal medications offer many advantages as they provide safe, fewer side effects, inexpensive, and effective medicines. Preclinical and clinical investigations of these plants should be examined to seek for offer new agents for the field in drug discovery [4]. The Viburnum tinus (*V.tinus*) is a shrub or small tree native to the Mediterranean region. The plant has fragrant, white, tiny flowers and drupe-type fruits with a metallic steely black dark blue color. The common name "laurustinus" is thought to refer to its leaves, which are similar to those of the bay tree (Laurus nobilis). It has been

*Corresponding author e-mail: fatmahassan.fs@gmail.com
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shown to contain a variety of biochemical components, including iridoids, coumarins, saponins, and flavonoids [5] and [6].

The genus Viburnum comprises over 230 species under family name Adoxaceae. Monoterpenes, iridoids, coumarins, flavonoids, several oleanane-type triterpenes and diterpenes have been isolated from other Viburnum species [7]. Traditional medicine has used the roots, stems, leaves, and mature fruits of these plants for their ability of detoxification and reducing fever, as well as promoting blood circulation and removing blood stasis [8]. Recent research reported that it has anti- tumor, anti-oxidant, hypoglycemic, neuroprotection, and antibacterial biological properties. It has also been used as an anti-inflammatory drug for the first time in over a decade [9].

The biological activity of *V.tinus* leaves different solvents extracts as anti-inflammatory, anticancer, and apoptotic agent were investigated in this research.

Experimental:

1. *Plant material:*

During the winter of 2019, leaves of Viburnum tinus *(V.tinus)* were taken from El-Zoharia Botanical Garden in El-Zamalek, Cairo, Egypt. Dr. Amal A. Hagag was nice enough to identify the plant leaves that were collected (Lecturer of Taxonomy, Ornamental Research Institute, El-Dokki, Cairo, Egypt). It is maintained in the herbarium of the medicinal chemistry department, Theodor Bilharz research institute (TBRI), Giza, Egypt, under Voucher specimens (Reg. No.: V-20).

2. *Extraction and fractionation procedure:*

The leaves of *V.tinus* were picked, air dried, and milled into a dry powder, which was then steeped in 95% methanol for one week at room temperature with daily shaking, then filtered. The organic solvent was evaporated under vacuum using a rotatory evaporator, providing a crude methanol extract. Pellet was washed numerous times with n-Hexane; the collected wash was then evaporated by a rotatory evaporator to get a n-Hexane fraction. The remaining pellet was washed numerous times with $CH_2Cl_2$and collected and rinsed for additional evaporation to produce the chloroform fraction, leaving a pellet of the MeOH fraction (Residue). Each extract was weighted in order to calculate the percent of the yield [10].

3. *Sample preparation:*

A stock solution for each extract (50 mg/ml) was prepared by dissolving 0.05 gm in 1 ml of 100% DMSO to be diluted to 1% DMSO with start concentration (500 µg/ml) for activity evaluation , samples aliquots kept in -20°c.

4. *Qualitative phytochemical analysis:*

Phytochemical ingredients such as flavonoids, terpenoids, tannins, phenolic compounds, coumarins, and carbohydrates were determined in various plant extracts:

4.1. **Test for flavonoids:**

The Aluminum chloride test was used for detection of flavonoids in different extracts; the extract sample (3 ml) was mixed with 4 ml of 1% $AlCl_3$ (Merck Chemical Co) in MeOH (Adwic Egypt) in a test tube. The presence of flavonoids is indicated by the yellow color [11].

4.2. **Test for terpenoids:**

For terpenoids detection, the Liebermann-Burchard test was used: 2 ml plant extract was mixed with 2 drops of chloroform (El-Nasr Pharmaceutical Chemicals Co), then 1-2 ml acetic anhydride was added (Sigma), followed by 2 drops of conc. H_2SO_4 (Merck Chemical Co) red, blue, followed by dark green color appears, confirming the presence of terpenoids [12].

4.3. **Test for tannin and phenolic compounds:**

The ferric chloride test was used to detect tannins and phenolic compounds; extract (0.5 g) was boiled in 20 ml distilled water in a test tube and then filtered; two drops of 5% $FeCl_3$ (Merck Chemical Co) were added to 1 ml filtrate; the appearance of brownish green or blue-black coloration or a dirty green precipitate indicated the presence of tannins and phenolic compounds [13].

4.4. **Test for saponins:**

Plant extract or dry power (0.5 g) was mixed in boiling distilled water (5 ml) in a test tube and allowed to cool to identify saponins via frothing (foam) generation. [14].

4.5. **Test for coumarins:**

To evaluate the presence of coumarins, a tiny test tube was filled with moistened plant extract (0.5 g) and coated with filter paper wet with 1 N NaOH (Sigma). The test tube was

immersed in boiling water for a few minutes. Coumarins were found as a yellow florescence when the filter paper was removed and inspected under UV light [15].

4.6. **Test for carbohydrates:**

Molisch's test is used for carbohydrate detection; for aqueous extract, two drops of alpha-naphthol solution were added followed by shaking and adding conc. H_2SO_4 (El-Nasr Pharmaceutical Chemicals Co **)** from the sides of the test tube. A violet ring is formed and the junction of the two liquids indicating the presence of carbohydrates [16].

5. Biological activity:

5.1. *Evaluation of anti-inflammatory activity:*

The anti-inflammatory activity of *V.tinus* leaves different extracts **Crude , CH_2CL_2**(fraction)**, n-Hexane**(fraction)**, MeOH** (fraction) were evaluated using the following assays Protein denaturation inhibition [[17];[18]], Proteinase inhibition [[19]] , Membrane stabilization [[20] and [21]] and Inflammatory cytokine inhibition [[22] ,[21] ; [23]] assays with modifications . Diclofenac potassium (Catafast 50mg) obtained from Novartis was used as reference drug.

- **Protein denaturation inhibition :**

Bovine serum albumin BSA (Sigma) 1 % (v/v) aqueous solution (450µl) was mixed with (50µl) extracts of various concentrations (500, 250, and 125 µg/ml) and incubated at 37^{o}C for 20 minutes, followed by heating at 65^{o}C for 20 minutes, cooling, and turbidity measurement at 660nm against a blank (d.H2o). the extract was compared to a reference drug (Diclofenac potassium).

Percentage of inhibition = (O.D. of control– O.D. of test) x 100/ O.D. of Control.

- **Proteinase (trypsin) inhibition:**

A reaction mixture of (25µl) 0.06 mg trypsin (Cegrogen), (50µl) 20mM Tris HCl buffer (pH 7.4) and (50µl) sample or reference drug at different concentrations (500:125 µg/ml), were incubated at 37°C for 5 min. 100 µl of 0.8% aqueous solution (BSA) was added and incubated for another 20 min. the reaction was terminated by adding (100 µl) 10% perchloric acid (Sigma), resulting in a cloudy suspension. The suspension was centrifuged at 10000 rpm for 15 minutes at 4°C. The absorbance of the supernatant was read at 210 nm against 10% perchloric acid as a blank.

Percentage inhibition = (O.D. of control– O.D. of product test) x 100 O.D. of Control

- **Membrane stabilization:**

I.Preparation of RBCs solution: 3 ml of blood was transferred to the EDTA tubes from a healthy human volunteer who has not taken any NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for 2 weeks before to the experiment.

The supernatant was collected and the pellet was washed three times with an equal volume of normal saline in each wash after the blood was centrifuged at 500 rpm for 5 minutes at 4°C. The supernatant was removed, and pellet (RBCs) was diluted with normal saline at a 1:10 ratio (10 % RBCs solution).

*II.Hemolytic toxicity :*the extract sample and reference drug different concentration as 500, 250,125 µg/ml were prepared and then 200µl of each concentrations were mixed with 50µl of (10% RBCS suspension) The mixture was incubated at 37°C for 30 min then centrifuged at 10,000 rpm for 3 min. The absorbance was measured at 490 nm and experiment was performed in triplicate. The percentage of hemolysis was calculated using the following equation :

Hemolysis % = [OD (sample) - OD (-ve)]/ OD (+ve) X 100.

Where,(+ve)1%triton x100 and (-ve) normal saline

III.Heat induced hemolysis: A reaction mixture was made out of an equal volume of extract sample or reference medication at varied concentrations of 500,250,125µg/ml, as well as a control using normal saline instead of sample. After 30 minutes in a water bath at 56°C, the mixture was cooled and centrifuged at 2700 rpm for 2 minutes. The absorbance was measured at 560 nm.

Membrane stabilization % = [OD (control) - OD (test)] / OD (control) × 100.

- **Interferon gamma (INF-ɤ) inhibition:**

I. Isolation and culture of PBMC:

Approximately 5 ml of blood from healthy donors was diluted in Dullbecco's phosphate

buffer saline (DPBS) (Cegrogen biotech ,H0500-540) in a 1:1 ratio, then gently added to 7.5 ml of diluted blood over 2.5 ml of Ficoll paque™ plus (obtained from Amersham Bioscience), and centrifuged at 400g for 30-40 minutes at 20^0C. After centrifugation, a layer of buffy coat (PBMC) mononuclear cells was collected, washed twice in PBS, suspended in 5 ml of RPMI media (Cegrogen biotech,E0500-380) viability was checked with trypan blue dye (Biowest, L0990) and cells were cultured on 24 tissue culture plates at a concentration of $5x10^5$ cells in RPMI media supplemented with 10% FBS(Cegrogen biotech, A0500-3210) 1% HEPES buffer 1M (Cegrogen biotech, H0100-620) and 1% antibiotic/antimycotic 100x (Cegrogen biotech, P0100-770) and incubated at 37^0C overnight.

II. Inflammation induction:

Cell suspensions were stimulated for inflammation with 10 µg/ml phytohemagglutinin (PHA) which was obtained from (GIBCO BRL, USA), and incubated for another 24-hour at 37^oC. Different extracts and reference drug concentrations (400, 200, 100, 50 µg/l) were then added to activated cells, followed by another overnight incubation at 37^oC. Finally, the media was collected, centrifuged at 400g for 30-40 minutes, and the supernatant was collected.

III. Enzyme Linked Immunosorbant Assay (ELISA) for INF-ɤ level detection:

The collected supernatant was used to estimate INF-ɤ level detection by specific ELISA kit (Invitrogen, code: EHIFNG) according to the manufacturer´s instruction and the absorbance was read at 492nm using ELISA reader Bio-Tek (Model ELX808IU).

5.2. *In-vitro anti-cancer activity:*

The anticancer activity of *V.tinus* leaves different extracts **Crude, CH_2CL_2, n-Hexane, MeOH** was evaluated by detection of cytotoxicity on cancerous cell lines e.g. HepG2, Caco2, MCF7 and on Vero as normal cell line using MTT colorimetric assay [24] and [25] with modification. Doxorubicin hydrochloride obtained from (Pfizer) was used as a reference drug.

- **Cell lines***:* Human hepatoma (HepG2) ATCC®No.HB-8065, epithelial human breast adenocarcinoma (MCF7) ATCC®No.HTB-22, epithelial human colon adeno carcinoma (Caco-2) ATCC®No.HTB-37, and adult African green Monkey Kidney epithelial cell (Vero) ATCC®No.CCL-81 were obtained from VACSERA, Egypt.
- **Cell cytotoxicity**: Malignant and normal cell lines were cultured in RPMI and DMEM (Cegrogen biotech, E0500-120) respectively, which was supplemented with 10% FBS, 1% HEPES, and 1% antibiotic/antimycotic, and incubated overnight at 37°C. About $10x10^3$ cells were plated on a 96-well tissue culture plate and incubated overnight at 37°C. Viburnum tinus extracts in various concentrations, starting at 200:12.5 µg/ml (1% DMSO), were added to cell culture and incubated overnight at 37°C. 15 µl of MTT powder (Sigma-Aldrich , M2003) (0.5 mg/mL) was added to each well and incubated for 4 hours at 37°C after removing the media and washing with phosphate buffer saline. 150 µL of DMSO was added to each well and agitated in a shaker to dissolve the formazan crystals, a reduced product of MTT. The optical density (OD) was measured at 540 nm.

  % Cell cytotoxicity = {(OD cell control -OD sample)/ OD cell control} ×100.

- **Selectivity index (SI):** Each sample's cytotoxicity on cancerous cells is an IC_{50} value, and on normal cells, the CC_{50} value is preferred. These values are the average of the concentrations of test agents that caused a 50% inhibition or cell death. This value was calculated by graphing % inhibition versus compound concentration.

Selective index (SI) =CC_{50} /IC_{50}

5.3. *Apoptosis evaluation:*

The induction of apoptosis by DNA fragmentation assay [26] and Real-time PCR detection [27] of some apoptotic markers was investigated in relation to the anti-cancer efficacy of *V.tinus* extracts.

- *DNA fragmentation:*

In a 24 well tissue culture plate, 50,000 HepG2 cells were used. After the cells had attained

confluence, they were treated with extracts at IC_{50} levels and incubated for 24 and 48 hours. The cells were rinsed with (PBS). Adherent cells were imaged before being trypsinized. DNeasy cell culture kit (QIAGEN: 13323) was used to extract DNA from obtained cells. Using a Mini SUB ™ gel electrophoresis cell (Bio-RAD, Richmond, CA, USA) , each 10µ l of DNA was mixed with 2 µl of loading buffer in a 0.8% agarose gel stained with 5.0 mg/ml ethidium bromide and run at 75 V for 60 minutes. To assess the size of the DNA fragments, a 1 kb DNA ladder (Genedirex, Cat: DM010-R500) was used as a marker and examined by Ultraviolet Transilluminator (model T 2201, Sigma Chemical USA, Co).

Quantitive Real time PCR analysis:

Total RNA was extracted from HepG2 cells using the QIAGEN (Cat. 74134) kit according to the manufacturer's instructions, and then reverse transcribed using the Thermo Scientific kit (Cat. K1621) to have cDNA. The Roche Light Cycler480 (96-well block) was used to evaluate the relative expression of the BAX and BCL2 genes using cDNA as a template (USA) and SYBR Green master mix (Transgen : AQ601-01). The following primers sequences were created using ncbi primer design according to [28] and retrieved from Biovision:

BCL2 forward primer:
5'GTGGAGGAGCTCTTCAGGGA3'
reverse primer:
5'AGGCACCCAGGGTGATGCAA3'.
BAX forward primer:
5'TTGCTTCAGGGTTTCATCCA3'
reverse primer:
5'CAGCCTTGAGCACCAGTTTG3'.
GAPDH forward primer:
5'GGCTCTCCAGAACATCAT3'
reverse primer:
5'CACCTGGTGCTCAGTGTA3'.

To determine fold change in target gene expression, the housekeeping gene GAPDH was utilized as a reference. Amplifications were performed with initial denaturation at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 10 s , annealing and extension at 60 °C for 1min.

The cycle threshold (Ct) values for target genes and the housekeeping gene were calculated and relative gene expression was measured using the $2^{\wedge-\Delta\Delta Ct}$ method [29].

6. Statistical analysis:

The Graph prism program was used to perform statistics, and data reported as mean ± standard deviation (SD) was examined using the ANOVA test. A statistically significant P value of 0.05 is accepted.

Results:

1.Extraction yield:

As showed in Table.1, the highly yield of V. tinus fractions from crude extract was of CH_2Cl_2 which is a good solvent for extraction.

Table (1): yield percent of different extracts from 2kg of *V. tinus* leaves.

Extract	Weight (gm.)	Yield %
Crude (*ex.*)	280	14
CH_2Cl_2 (*fr.*)	96.31	4.8
n-Hexane (*fr.*)	39.6	1.98
MeOH (*fr.*)	14.5	0.73

2. Qualitative phytochemical analysis:

Phytochemical qualitive analysis of *V.tinus* leaves found that its different extracts contained flavonoids, saponins, terpenoids, tannins, and coumarins, among other phytochemicals as in Table (2).According to the results, n-Hexane extract contains the highest flavonoids, tannins, phenolic compounds, saponins, and coumarins levels whereas MeOH extract and CH_2Cl_2 contain the most terpenoids, and Crude contains the least amount of all phytochemicals.

3. Biological activity:

3.1. Evaluation of anti-inflammatory activity:

As presented in **Table (3)**; the maximum protein denaturation inhibition% of *V.tinus* extracts at concentration 500 µg/ml was of MeOH extract (96.70 ± 2.30) followed by n-Hexane extract (94.60 ± 3.90) and other extracts seems to be similar.

Table (2): Phytochemical analysis of different *V.tinus* extracts

Phytochemical	Extracts			
	Crude	CH_2Cl_2	n-Hexane	MeOH
Flavonoids	+	++	+++	+++
			+	+
Saponins	+	+++	+++	+++
Terpenoids	++	+++	++	+++
Tannins/	+	++	+++	++
phenolic acids				
Coumarins	++	++++	+++	+
Carbohydrates	++	++	+++	++

While; (+) represent low presence, (++) moderate presence, (+++) highly presence, and (++++) very high presence

At a concentration of 500 µg/ml of plant extracts, the highest suppression of proteinase of plant extracts was achieved by n-Hexane extract (88.5 ± 0.50) followed by MeOH extract (76.9 ± 0.45), CH_2Cl_2 extract (74.3 ± 5.03), and finally crude extract (47.4 ± 0.36).

The degree of hemolytic activity used to evaluate toxicity and it can be evaluated by the rate of RBC_s membrane rapture as following up to 10% is nontoxic, up to 49% is slightly toxic, up to 90% is considered toxic, and from 90% is categorized as a highly toxic, According to the data, all extracts at concentrations ranging from 500 to 125 µg/ml have no toxicity on RBCs membranes, with the exception of the MeOH extract followed by CH_2Cl_2 which has a minor level of toxicity 21.8 ± 5.30 and 16.4 ± 2.30 respectively at 500 µg/ml. on the other hand , when RBC_s induced hemolysis by heating the maximum inhibition of hemolysis at concentration 500 µg/ml was of n-Hexane extract (73.2 ± 1.58) followed by MeOH extract (70.5 ± 0.50).

Table (4) shows the suppression of inflammatory cytokine levels as measured by interferon gamma (INF-ɤ) utilizing various extracts on inflammation- induced lymphocytes.

At the concentration of 400 µg/ml, n-Hexane and MeOH extracts inhibited interferon gamma (INF- ɤ) levels the highest (100.0±0.00) followed by CH_2Cl_2 extract (80.7±6.02), and finally crude extract (48.9± 8.27).

3.2. Evaluation of Anti-cancer activity:

The *V.tinus* different extracts were tested for cytotoxic activity on cancerous cell lines as HepG2, Caco2, and MCF7and normal cell line as Vero at various concentrations from200 µg/ml to 12.5 µg/ml and cytotoxic percent detected by MTT assay. At a concentration of 200µg/ml, the n-Hexane extract demonstrated the most cytotoxic activity in HepG2 80.0±1.30% followed by 76.9±1.35% in MCF7 and 63.1±1.00% in Caco2 compared to 47.5 ± 1.69 % in Vero as reported in Table (5), while the crude extract showed the least IC_{50} of 29.7±2.5 and 4.67 ± 0.32 µg/ml in HepG2,29.1±0.59 and 40.6 ±1.60 µg/ml in Caco2, and 75.3±4.70 and 58.1 ± 1.10 µg/ml in MCF7 compared to 305.3 ± 56.5 and 121.4 ±27.01 µg/ml in Vero respectively .

As SI measures the extract's differential activity, Table (5) shows that the MeOH extract had the highest selectivity index (SI) in HepG2 and Caco2 45.9 and 16.3 respectively, compared to Dox 25.9 in HepG2 and 2.9 in Caco2, and that n-Hexane had the highest level in MCF7 10.4 compared to Dox 2.9.

3.3. Evaluation of apoptotic activity:

As showed in **Figure (4),** untreated HepG2 cells had a normal round and polygonal morphology, however after 24 and 48 hours of treatment with various extracts at IC50, the cells began to change morphology, becoming somewhat elongated and smaller and became detached. The presence of inter-nucleosomal DNA cleavage on agarose gel electrophoresis verified apoptosis. Cells treated with IC_{50} value for 48 hours revealed fragmented DNA on agarose gel electrophoresis, showing the anti-proliferative activity of n-Hexane, followed by MeOH, CH_2Cl_2, as showed in **Figu r e (5).**

Table (3): *In-vitro* anti-inflammatory activity of different *V. tinus* extracts comparing to Diclofenac potassium as reference drug.

Sample	Conc. µg/ml) (	Protein denaturation Inhibition%	Proteinase Inhibition %	Membrane stabilization	
				Hemolytic toxicity%	Heat induced hemolysis inhibition%
Crude (*ex.*)	500	85.25 ± 2.80^{ns}	47.4 ± 0.36****	03.0 ± 0.03****	22.5 ± 0.50****
	250	61.65 ± 1.40^{ns}	31.3 ± 0.31*	02.4 ± 0.50****	20.4 ± 0.53****
	125	53.55 ± 1.80$^{\$}$	12.4 ± 0.45$^{\$\$\$\$}$	00.0 ± 0.00$^{\$\$\$\$}$	18.5 ± 0.51$^{\$\$\$\$}$
CH_2Cl_2 (*fr.*)	500	85.35 ± 0.43 ^{ns}	74.3 ± 5.03****	16.4 ± 2.30****	38.7 ± 1.10*
	250	80.25 ± 0.65^{ns}	46.6 ± 3.00^{ns}	09.1 ± 0.94****	29.6 ± 0.55^{ns}
	125	68.45 ± 0.51 ^{ns}	21.3 ± 0.65 ^{ns}	00.0 ± 0.00$^{\$\$\$\$}$	24.5 ± 0.50$^{\$\$\$\$}$
n-Hexane (*fr.*)	500	94.60 ± 3.90 ^{ns}	88.5 ± 0.50****	07.3 ± 0.75****	73.2 ± 1.58****
	250	92.40 ± 4.10^{ns}	80.6 ± 5.90$^{####}$	04.8 ± 0.23$^{####}$	71.9 ± 1.00$^{####}$
	125	85.05 ± 8.10 $^{\$}$	23.1 ± 0.40 ^{ns}	01.2 ± 0.04$^{\$\$\$\$}$	54.3 ± 0.57$^{\$\$\$\$}$
MeOH (*fr.*)	500	96.70 ± 2.30 ^{ns}	76.9 ± 0.45****	21.8 ± 5.30****	70.5 ± 0.50****
	250	90.35 ± 0.40^{ns}	38.7 ± 0.32$^{\#}$	13.9 ± 1.56$^{####}$	58.8 ± 1.02$^{####}$
	125	84.85 ± 0.17$^{\$}$	26.6 ± 0.45$^{\$}$	07.3 ± 0.36$^{\$\$\$\$}$	32.3 ± 0.57$^{\$\$\$\$}$
Diclofenac potassium	500	89.50 ± 3.50	66.1 ± 0.26	58.2 ± 11.10	40.9 ± 0.85
	250	85.00 ± 4.20	42.7 ± 0.44	54.5 ± 9.34	30.7 ± 0.68
	125	58.70 ± 18.0	32.0 ± 0.55	44.2 ± 18.20	29.1 ± 1.10

Values are presented as mean ± SD, test performed triplicate for each group. **** Significantly different from the reference drug conc 500 µg/ml at P<0.0001, $^{####}$ Significantly different from the reference drug conc 250 µg/ml at P<0.0001, $^{\$\$\$\$}$ Significantly different from the reference drug conc 125 µg/ml at P<0.0001, *Significantly different from the reference drug conc 500 µg/ml at P<0.01 , $^{\#}$ Significantly different from the reference drug conc 125 µg/ml at P<0.01 , $^{\$}$ Significantly different from the reference drug conc 125 µg/ml at P<0.01and ^{ns}Significantly different from the reference drug at P>0.05.

Table (4): Enzyme Linked Immunosorbant Assay (ELISA) for INF- ɤ level inhibition %:

Sample	Conc (µg/ml)	OD	Conc INF-ɤ (µg/ml)	Inhibition%
CC (-ve control)	-----	0.0004 ± 0.002	00.0 ± 0.00	----------
INF-ɤ(+ve control)	-----	0.128 ± 0.005	158.4±8.40	00.0
Crude (*ex.*)	400	0.086 ± 0.003	87.3 ± 5.09	48.9 ± 8.27****
	200	0.099 ± 0.004	109.5 ± 6.66	30.8 ± 4.20****
	100	0.119 ± 0.004	142.8 ± 6.01	9.8 ± 3.77****
	50	0.129 ± 0.003	160.0 ± 5.09	0.83± 1.44****
CH_2Cl_2 (*fr.*)	400	0.052 ± 0.006	30.5 ± 9.53	80.7 ± 6.02**
	200	0.061 ± 0.002	45.4 ± 3.69	71.3 ± 2.35**
	100	0.093 ± 0.009	100.1 ±14.38	36.7± 9.09**
	50	0.117 ± 0.004	138.7 ± 6.81	12.3 ± 4.31**
MeOH (*fr.*)	400	0.012 ± 0.004	00.0 ± 0.00	100 ± 0.00^{ns}
	200	0.025 ± 0.003	00.0 ± 0.00	100 ± 0.00 ^{ns}
	100	0.035 ± 0.001	2.3 ± 0.96	98.5 ± 0.57 ^{ns}
	50	0.057 ± 0.005	39.5 ± 8.82	75.0 ± 5.61 ^{ns}
n-Hexane (*fr.*)	400	0.015 ± 0.004	00.0 ± 0.00	100 ± 0.00 ^{ns}
	200	0.044 ± 0.003	17.2 ± 5.42	89.0 ± 3.40 ^{ns}
	100	0.058 ± 0.003	41.1 ± 4.46	74.0 ± 2.81 ^{ns}
	50	0.068 ± 0.002	57.7 ± 3.25	63.5 ± 2.10 ^{ns}
Diclofenac potassium	400	0.021 ± 0.003	00.0 ± 0.00	100 ± 0.00
	200	0.037 ± 0.004	6.20 ± 5.65	96.0 ± 3.60
	100	0.055 ± 0.004	36.0 ± 7.00	77.2 ± 4.45
	50	0.088 ± 0.005	91.8 ± 8.31	42.0 ± 5.23

Values are presented as mean ± SD, test performed triplicate for each group. **** Significantly different from the reference drug at P<0.0001, **Significantly different from the reference drug at P<0.001 and ^{ns}Significantly different from the reference drug at P<0.05.

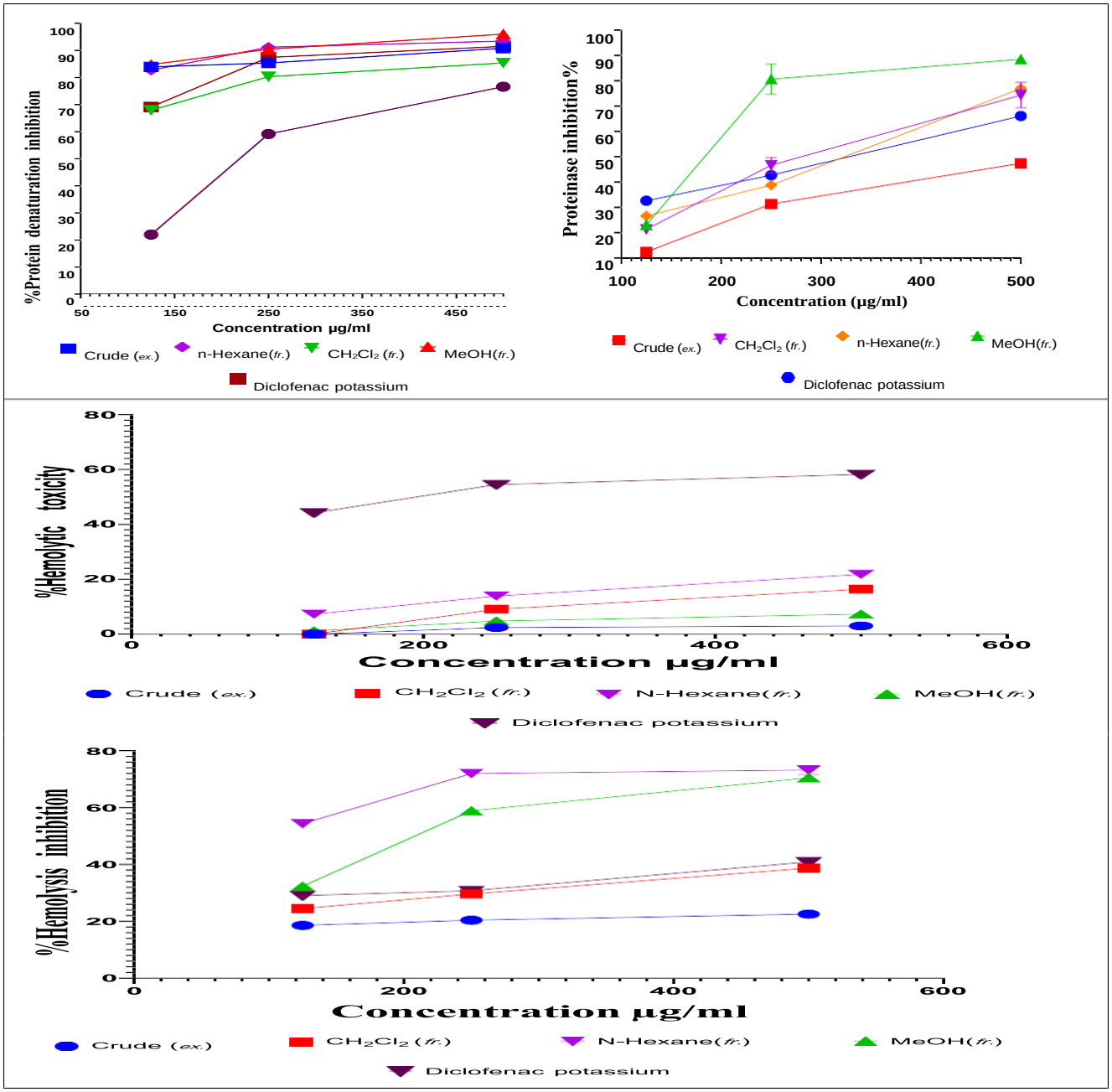

Figure (1): *In-vitro* anti-inflammatory activity of different *V.tinus* extracts comparing to Diclofenac as reference drug.

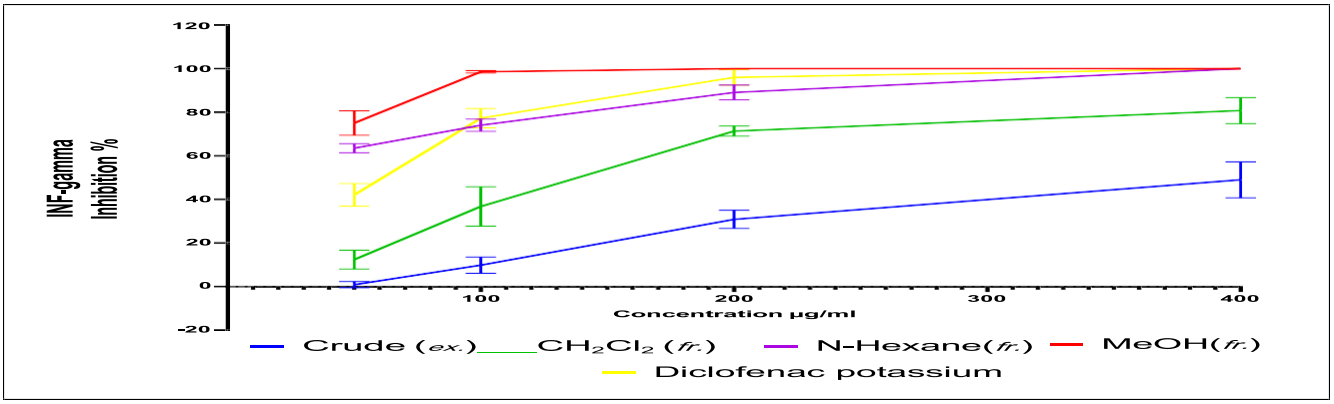

Figure (2): Different *V.tinus* extracts inhibition percent on INF- ɣ level.

Table (5): *In-vitro* cytotoxic percent, IC_{50} and SI of *V.tinus* extracts on HepG2, Caco2, MCF7, and Vero cell lines comparing to Doxorubicin as reference drug:

1. $HepG_2$	Conc.(µg/ml)					IC_{50} (µg/ml)	SI
	200	100	50	25	12.5		
Crude (*ex.*)	74.9 ± 0.56	65.6 ±0.97	54.6 ± 0.73	41.7 ± 0.95	34.2 ± 1.70	**35.6 ± 3.13******	**42.4**
MeOH (*fr.*)	66.3 ± 0.40	58.0 ± 1.30	45.8 ± 2.80	18.5 ± 1.00	12.4 ± 1.90	**68.3 ± 3.50******	**45.9**
CH_2Cl_2 (*fr.*)	76.8 ± 0.60	74.4 ±1.20	74.0 ± 0.50	52.0 ± 0.60	50.0 ± 1.50	**12.9 ±0.27****	**34.2**
n-Hexane (*fr.*)	80.0 ± 1.30	69.0 ± 3.00	65.0 ± 0.95	36.0 ± 4.00	35.5 ± 1.00	**29.7 ± 2.58******	**10.2**
Dox	85.7± 0.86	85.2 ±0.90	84.6 ± 1.50	74.8 ± 1.60	64.8 ± 1.60	**4.67 ± 0.32**	**25.9**
2.$CaCO_2$							
Crude (*ex.*)	41.4 ± 0.37	38.9 ± 0.23	32.2 ± 0.36	29.6 ± 0.27	26.1 ± 0.94	**880.31 ± 45.60******	**1.7**
MeOH (*fr.*)	34.8 ± 0.49	31.6 ± 1.02	27.5 ± 2.23	23.0 ± 0.97	15.5 ± 1.89	**1648.54±08.40******	**16.3**
CH_2Cl_2 (*fr.*)	58.6 ± 1.07	36.6 ± 1.40	28.7 ± 1.63	23.4 ± 0.70	21.8 ± 1.16	**168.1 ±5.90^{ns}**	**2.3**
n-Hexane (*fr.*)	63.1 ± 1.00	62.1 ± 0.47	60.4 ± 0.20	49.6 ± 0.70	28.5 ± 1.06	**29.1 ± 0.59^{ns}**	**4**
Dox	50.3 ± 3.15	45.4 ± 0.50	42.1 ± 2.80	31.4 ± 1.65	28.5±0.76	**40.6 ±1.60**	**2.9**
3.MCF7							
Crude (*ex.*)	42.5 ± 1.32	32.6 ± 1.75	24.0 ± 1.10	18.3 ± 1.17	08.7 ± 0.40	**411.2 ± 3.30******	**3.6**
MeOH (*fr.*)	51.6 ± 1.11	32.2 ± 1.59	19.7 ± 0.65	17.2 ± 1.81	08.1 ± 1.40	**192.5 ± 4.20******	**1.9**
CH_2Cl_2 (*fr.*)	71.6 ± 1.77	54.7 ± 1.55	43.6 ± 1.32	28.7 ± 0.90	23.0 ± 1.17	**69.9 ± 1.80******	**6.3**
n-Hexane (*fr.*)	76.9 ± 1.35	73.4 ± 1.15	66.9 ± 0.45	62.5 ± 1.59	51.3 ± 1.30	**75.3 ± 4.70******	**10.4**
Dox	74.2 ± 3.16	63.5 ± 2.81	47.4 ± 0.55	41.8 ± 1.70	40.4 ± 0.72	**58.1 ± 1.10**	**2.9**
4.Vero							
Crude (*ex.*)	33.0 ± 0.88	28.2 ± 1.82	21.3 ± 1.52	------	------	**1508 ± 388.8****	
MeOH (*fr.*)	34.4 ± 1.66	32.3 ± 1.95	28.6 ± 3.58	------	------	**3140.6 ± 801******	
CH_2Cl_2 (*fr.*)	41.5 ± 1.40	31.4 ± 2.24	25.5 ± 1.50	------	------	**441.8 ± 39.9^{ns}**	
n-Hexane (*fr.*)	47.5 ± 1.69	40.9 ± 1.85	37.6 ± 3.05	------	------	**305.3 ± 56.5^{ns}**	
Dox	65.5 ± 2.20	45.2 ± 6.65	29.0 ± 1.73	------	------	**121.4 ±27.01**	

Values are presented as mean ± SD, test performed triplicate for each group. **** Significantly different from the reference drug at P<0.0001, *** Significantly different from the reference drug at P<0.001,** Significantly different from the reference drug at P<0.01,^{ns}Significantly different from the reference drug at P<.0.05.

Apoptosis (type of planned cell death) characterized by cytoplasm condensation and deformation of the plasma membrane, which leads to the fragmentation of nuclear DNA into multiples of 200 bp. Some phytochemicals can stop tumor cells from growing, however not all of them can cause apoptosis some phytochemicals can stop tumor cells from growing, however not all of them can cause apoptosis. The presence of inter-nucleosomal DNA cleavage on agarose gel electrophoresis verified apoptosis. Cells treated with IC_{50} value for 48 hours revealed fragmented DNA on agarose gel electrophoresis, showing the anti-proliferative activity of n-Hexane, followed by MeOH, CH_2Cl_2, as showed in Figure(5).

The mRNA expression of BAX and BCL-2 genes was investigated using qRT-PCR. In this investigation, GAPDH as the endogenous gene was chosen as a reference gene and variations in expression levels after 4 and 24 hours after treatment of $HepG_2$ with different *V. tinus* extracts IC_{50} values were compared and investigated. With time, the relative quantification (RQ) levels of the BAX gene increased while the level of BCL-2 gene expression decreased as showed in (Table 6).

As shown in Table (6) and Figure (6), the n-Hexane extract increased BAX levels higher than other extracts after 4 hours by 2.6 times comparing to control, and continued to increase to 5.8 folds than normal after 24 hours. While in the other hand the BCL-2 level was raised 2.6 fold more than normal after 4 hours of treatment and decreased till normal range after 24 hours of treatment. The BAX/BCL-2 ratio after 4 hours of treatment of extracts shows non-significant effect than normal cells in exception of CH_2Cl_2 extract which significantly increased than normal, while after 24 hours of treatment the n-Hexane shows a highly significant increase of the ratio followed by CH_2Cl_2 and MeOH.

Discussion:

The *V.tinus* leaves extract contains valuable phytochemicals such as acylated iridoids (viburtinoside A and B) , coumarin sophoroside, diethyl ester of nicotinic acid along with, triterpenoidal saponins, and flavonoids (nobiletin, afzelin, quercitrin, isoquercitrin, hyperin, and rutin), flavonol glycosides, and oleanolic acid which were quantified for the first time in this genus and species according to our previous studies reported by Mohamed et al. 2005 .Based on *Viburnum* species' use in folk medicine as diuretic, antispasmodic, and sedative agents, this research has contributed in the identification of *V. tinus* 'leaves different extracts **Crude**, **CH_2Cl_2**, **n-Hexane** and **MeOH** (Residue**)** potential therapeutic actions. Qualitative phytochemical screening was used as a preliminary step which enhanced the biological activities such as anti-inflammatory, anti-cancer, and apoptotic induction evaluation in vitro as predictors of *V.tinus* significance. According to the results of phytochemical constituents screening as flavonoids, saponins, tannins, and phenolic compounds, it was found that n-Hexane.extract has a sufficiently high level of these phytochemicals followed by MeOH, CH_2Cl_2, and finally crude extract. Though the n-Hexane extract has a moderate level of terpenoids and coumarins, the CH_2Cl_2 has the highest level. This data is in agreement with Muhammad Alam, 2012, who reported that the crude extract and chloroform fraction of *V. grandiflorum* root was not active, while n-hexane fraction was the most active one biologically.When an inflammatory disorder occurs, the lysosomal granules in leukocyte neutrophils release enzymes (proteinases) that are associated with inflammatory disorders, causing denaturation of cell proteins and loss of biological activities. Thus, agents that can inhibitthese proteinases and minimize protein denaturation can help prevent inflammatory symptoms and complications. The membrane of human erythrocytes (RBC_S) is similar to that of lysosomes; any stability of these membranes against inflammation increases anti- inflammatory function [32] and [18].In the present study, we found that *V.tinus* different extracts as the following order n-Hexane > MeOH>CH_2Cl_2 >Crude show inhibition of proteinase activity, protein denaturation inhibition, and increased membrane stabilization with minor toxicity on RBCs compared to the commercial drug Diclofenac potassium, in addition to content of flavonoids , terpenoids and saponins which reported to have anti-inflammatory activity in accordance with [17] .In response to inflammation ,chemical signaling activate immune cells such as lymphocytes by launching a cascade of mediators such as interferongamma (IFN-γ); pro-inflammatory cytokine that amplify the release of IL-1 and TNF that promoting the inflammatory process [33] and [34].

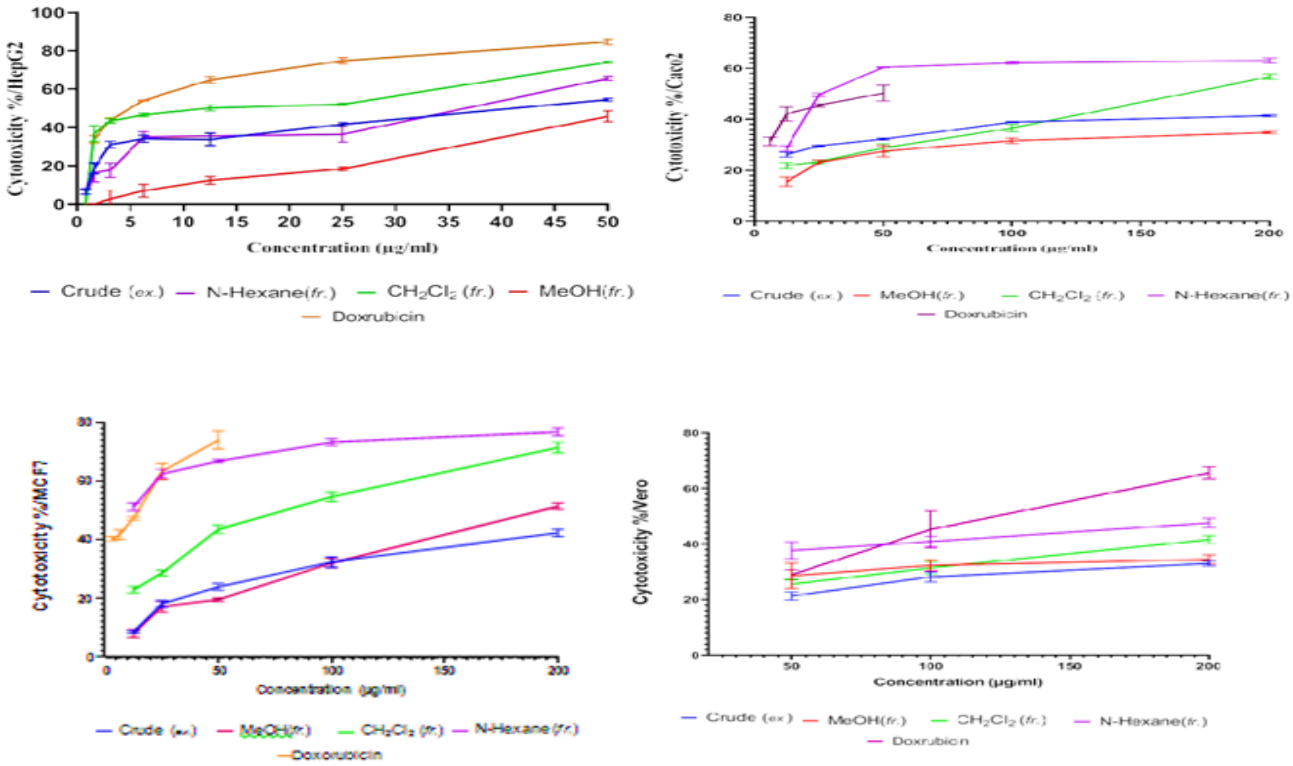

Fig (3): cytotoxic activity of different extracts.

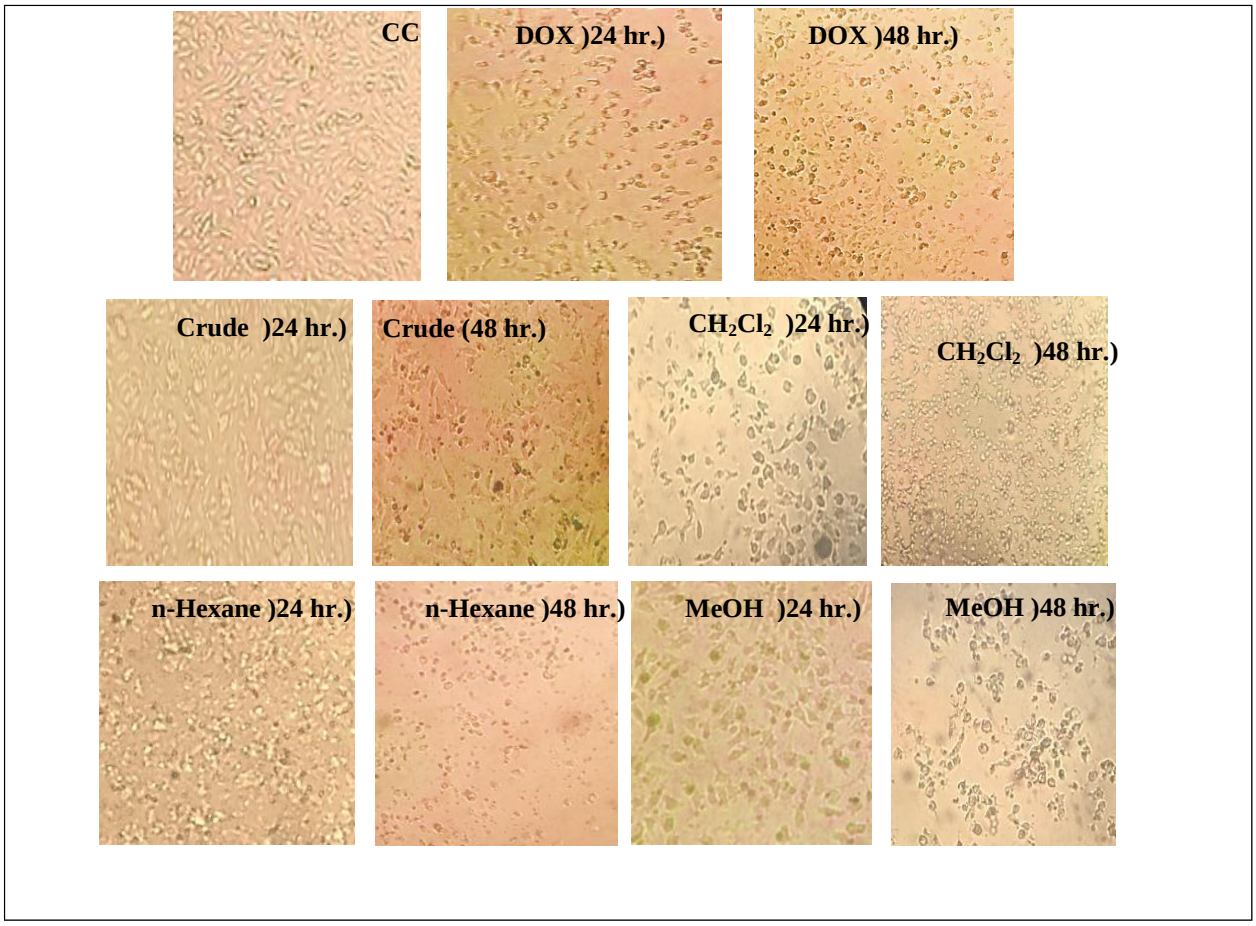

Figure (4): Effects of *V.tinus* different extracts on $HepG_2$ cell morphology treated with IC_{50} concentration after 24:48 hr., as observed under light microscope at magnification 40x.

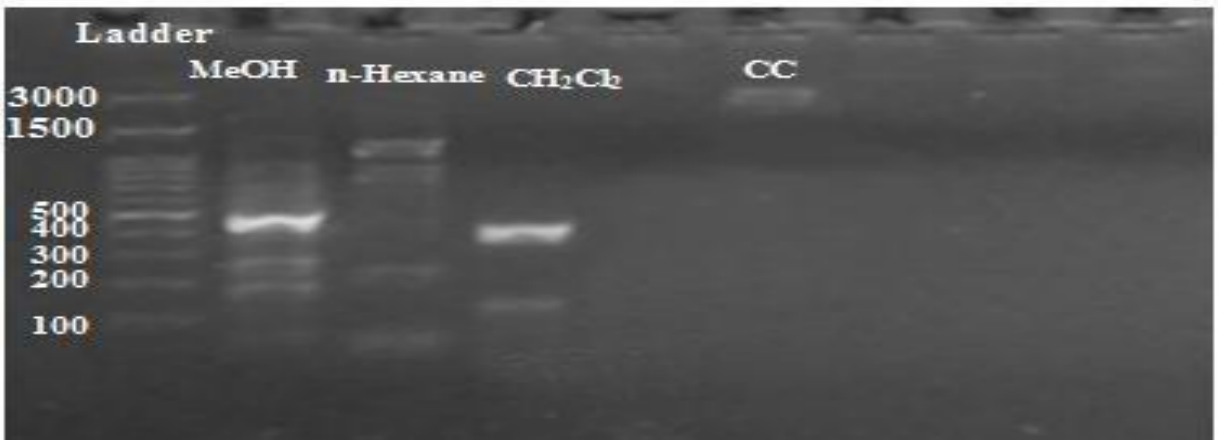

Figure (5): Gel electrophoresis showed DNA laddering formation in of different *V.tinus* extracts IC_{50} concentration after 48 hours of treatment during DNA fragmentation assay in 0.8% agarose gel.

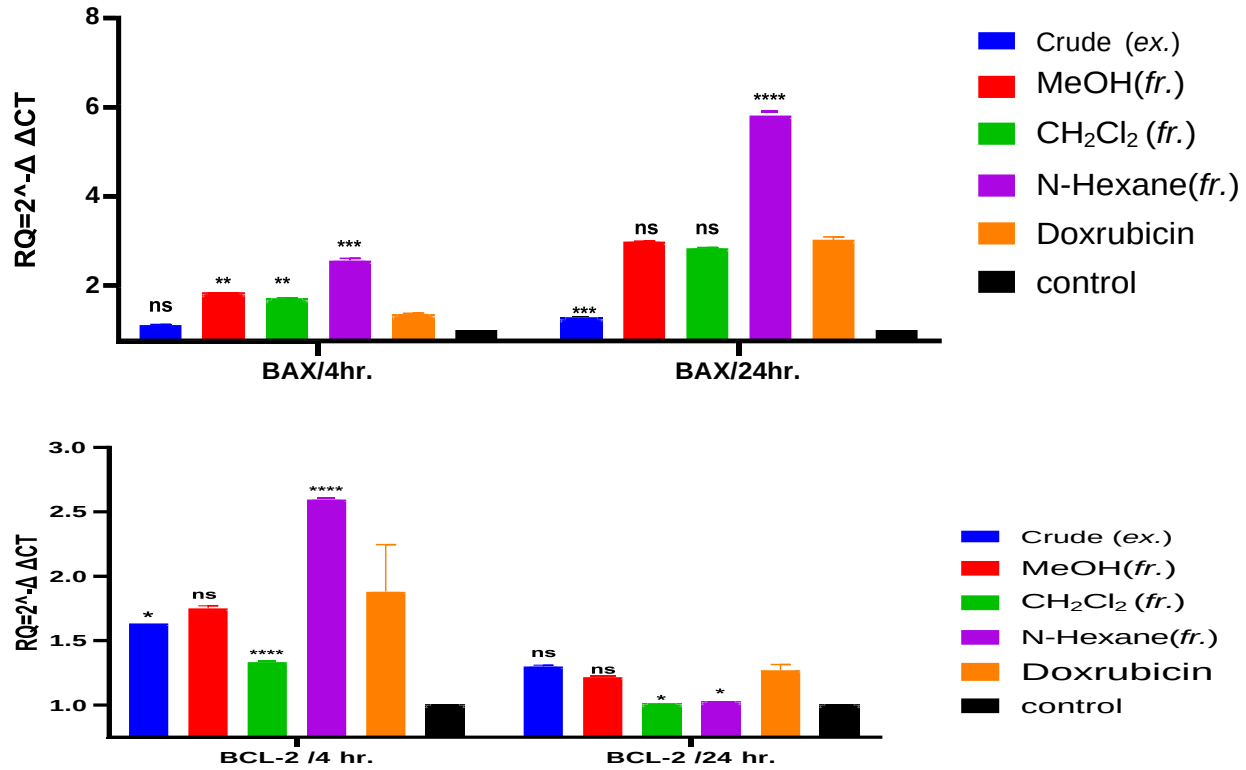

Figure (6): The mRNA expression level of apoptotic related genes (BAX, BCL-2) in $HepG_2$ cells induced by *V.tinus* different extracts IC_{50} after 4 and 24 hr.

Table (6): mRNA expression level (RQ =2^-ΔΔCT) of apoptotic genes in different *V.tinus* extracts

	BAX		BCL-2		BAX/BCL-2 ratio	
	4 hrs.	**24 hrs.**	**4 hrs.**	**24 hrs.**	**4 hrs.**	**24 hrs.**
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.000
Crude (ex.**)**	1.11 ± 0.01^{ns}	1.27 ± 0.02***	1.63 ± 0.03*	1.30 ± 0.01^{ns}	0.68 ± 0.015*	0.97 ± 0.013^{ns}
CH_2Cl_2(fr.**)**	1.70 ± 0.01**	2.84 ± 0.01^{ns}	1.33 ± 0.00^{ns}	1.01 ± 0.00^{ns}	1.28 ± 0.001*	2.80 ± 0.012**
MeOH (fr.**)**	1.83 ±0.00**	2.99 ± 0.00^{ns}	1.75 ± 0.02****	1.22 ± 0.01*	1.04 ± 0.007^{ns}	2.45 ± 0.020**
n-Hexane(fr.**)**	2.56 ± 0.05***	5.82 ± 0.01****	2.59 ± 0.01****	1.03 ± 0.00*	0.98 ± 0.023^{ns}	5.65 ± 0.026****
DOX	1.35 ± 0.02	3.03 ± 0.06	1.88 ± 0.36	1.27 ± 0.04	0.72 ± 0.120*	2.38 ± 0.130**

Values are presented as mean ± SD, test performed triplicate for each group. ****Significantly different from the reference drug at P<0.0001, ***Significantly different from the reference drug at P<0.001,**Significantly different from the reference drug at P<0.01,^{ns}Significantly different from the reference drug at P<0.05.

While IFN- may be targeted and blocked, it reduces inflammatory response. The n-Hexane and MeOH extracts show a decreasing level of IFN- γ in inflammatory stimulated lymphocytes due to their phytochemical constituents of saponins and phenolic compounds in accordance with [35], which has shown that these phytochemicals have an inhibitory effect on inflammatory cytokines. All of these support that *V.tinus* leaves different extracts have potential anti-inflammatory activity.

The scientific literature demonstrated that *Viburnum* species are rich in biologically active phytochemicals as phenolic compounds, flavonoids, iridoids and coumarins, which are particularly characterized by cancer prevention and anti-proliferative activity against numerous human cancer cell lines as reported by [36] and [37].

In the current study, *V.tinus* leaves crude extract and its fractions n-Hexane, MeOH, and CH_2Cl_2were evaluated for their cytotoxic effect on cancer cell lines; liver carcinoma ($HepG_2$), breast cancer (MCF7), and colon cancer (Caco2), and normal cell lines (Vero). The result shows that the different extracts exhibit anti-proliferative activity against cancerous cell lines in the following order: $HepG_2$>MCF7>Caco2.

From all extracts, n-Hexane is the most active one due to its high content of phenolic compounds and terpenoids which affect cell proliferation these results are in accordance with [38] who reported that triterpenoidal compounds found in *V. odoratissimum* showed remarked cytotoxic activity on $HepG_2$ and A549 cell lines. Also, the results are agrrement with [39]who reported that phenolic compounds found in in *V.opulus* effects on metabolic pathway of $HepG_2$ via AMPK pathway.

The powerful anti-tumor agent should eliminate cancer cells without any negative impacts possible on normal cells by what described as selective index (SI).The higher the SI value, the more selectivity it is. The SI value of more than 10 was thought to be anti-cancer, whereas a value of less than 2 was assumed to be hazardous [20], In the current data, MeOH has the highest selective index on HepG2, followed by crude extract and CH_2Cl_2, with n-Hexane having the lowest, while MeOH has the highest selective index in Caco2, followed by n-Hexane, crude extract, and CH_2Cl_2, while n-Hexane has the highest selective index against MCF7, followed by CH_2Cl_2, crude extract, and finally MeOH. All of the extracts have a greater SI, indicating that various extracts have distinct anticancer effectiveness on various cell types.

Apoptosis as a programming cell death can be induced by anti-cancer agents in tumor cells. Apoptosis begins with some morphological changes as cells begin to be shrinking and its outlines become convoluted forming extensions or buds from its cell membrane, in addition to nucleus damage and condensation of its chromatin [40] During apoptosis, cells nuclear membrane deformed and its content was destroyed as genomic DNA became fragmented into small fragments of 150 bp multiplex [41]. In this study, the effectiveness of *V.tinus* leaves different extracts on malignant cell line is accomplished through the induction of apoptosis and cell death to evaluate exactly how active agents behave to allow understanding how they can be used to treat cancer.

According to these results, n-Hexane is the most active extract which appears on cell morphology and confirmed by DNA laddering as $HepG_2$ extracted DNA after 48 hours incubation found fragmented in multiplex of 150 bp followed by MeOH and finally CH_2Cl_2.These data are attributed to the highest content of phenolic compounds in accordance with [42] who reported that *V.opulus* methanolic extract induces DNA damage, apoptosis, and cytotoxicity in a dose-dependent manner in cancer cells due to its phenolic compounds content.

The biochemical cleavage of cell genome and degradation of cellular proteins occurring during apoptosis resulted from apoptotic proteins activation either through extrinsic or intrinsic mitochondrial pathways. In the intrinsic pathway of apoptosis, the anti-apoptotic (BCL-2) and pro-apoptotic (BAX) proteins control and set the threshold for maintaining the death process in the intrinsic pathway of apoptosis, the BAX promotes cell death by permeabilizing the mitochondrial outer membrane, while BCL-2 prevents apoptosis by inhibiting BAX [43]. The

quantitative RT-PCR analysis of *V.tinus* extracts' effects on the HepG2 cell line shows that BAX gene expression increases while BCL-2 decreases. The BAX/BCL-2 ratio is used to predict cell apoptosis and its ability to influence tumor growth and aggressiveness. Lower values of this ratio indicate apoptosis resistance [43]. In these data, the relative quantification of BAX gene expression increased, while BCL-2 was inhibited in the following order: n-Hexane, MeOH, CH2Cl2, and crude. The BAX/BCL-2 ratio of the different extracts by time of treatment revealed (increased) susceptibility to apoptosis, which is consistent with [44], who reported that *V. negundo* has growth-inhibitory, apoptotic, and anti-cancer effects.

Conclusion:

The viburnum genus has been widely used in folk medicine; the *viburnum tinus* one member of this family and our previous study on its crude extract demonstrate its vital phytochemicals. the crude extract and its fractionated extracts (CH_2Cl_2, n-Hexane, MeOH) was evaluated for its therapeutic activity .In this study found that n-Hexane consider the most active extract of *V. tinus* leaves as anti-inflammatory, anti-cancer and apoptotic activity. These results assumed the potential therapeutic activity of *viburnum tinus* leaves extracts and put the basic for further studies on its bioactive compounds and it application in pharmaceutical purposes.

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